INFLUENCE OF COPPER ON RESISTANCE OF *LUMBRICUS TERRESTRIS* TO BACTERIAL CHALLENGE

Carla Stull Simmons, B.A.

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APPROVED:

Arthur J. Goven, Major Professor
Mark S. Shanley, Committee Member
Barney J. Venables, Committee Member
Earl G. Zimmerman, Chairman of the Department of Biological Sciences
C. Neal Tate, Dean of the Robert B. Toulouse School of Graduate Studies

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Earthworms, *Lumbricus terrestris*, were challenged orally and intracoelomically with two bacterial species, *Aeromonas hydrophila* and *Pseudomonas aeruginosa*, and mortality rates were observed. Neither were found to be particularly pathogenic at injected doses of up to 10^8 bacteria per earthworm. The influence of Cu^{++} (as $CuSO_4$) on the earthworm's response to bacterial challenge was investigated by exposing earthworms to sublethal levels of Cu^{++} prior to bacterial challenge. Exposure at sublethal concentrations up to 3 $\mu g/cm^2$ did not have a pronounced influence on host resistance to challenge as measured by earthworm mortality. Cu^{++} increased the earthworm's ability to agglutinate rabbit erythrocytes, indicating that Cu^{++} exposure caused coelomocyte death, autolysis and release of agglutinins into the coelom, possibly explaining resistance to bacterial challenge.

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INTRODUCTION

Chemicals found as contaminants in aquatic and terrestrial environments pose immunotoxic risk to public (human) and environmental (wildlife, range animal, and livestock) health. Assessing these risks is central for setting regulatory standards and requirements for clean-up strategies that ensure safety to humans and other organisms. Cost effective, socially non-controversial surrogate bioassay systems based on a biological function homologous to most metazoan animals would be invaluable to regulators, public health officials, and those economically or legally responsible for evaluating adverse effects of chemicals.

Previous work by the Environmental Effects Research Group (EERG) [1] at the University of North Texas (UNT) has resulted in the establishment of a suite of immunoassays using the earthworm, *Lumbricus terrestris*, for assessing immunotoxic risk of chemicals. Much of this research is based on the phagocytic and inflammatory responses mediated by immunoactive earthworm coelomocytes, cells morphologically and functionally homologous to vertebrate leukocytes, before and after in vivo and in vitro exposure to immunotoxicants. This model has been used to assess the toxic potential of many chemicals to vertebrates.

To validate the earthworm model as a surrogate companion to the National Toxicology Program's (NTP) [2] mouse model, chemical induced suppression of the

phagocytic and inflammatory responses must be correlated with reduced host resistance to infection by disease-producing organisms. This research is designed to begin that task. Herein, I describe a study in which I attempt to identify a microbial organism for use as a *L. terrestris* pathogen in a biomarker assay to determine the immunosuppressive potential of chemicals. My specific objectives were to (1) assess the pathogenicity of *Aeromonas hydrophila* and *Pseudomonas aeruginosa* to the earthworm *L. terrestris*, (2) assess the influence of Cu^{++} exposure on the pathogenicity of these organisms in earthworms, and (3) determine the effect of Cu^{++} on an antibacterial humoral immune function, agglutination.

I chose *A. hydrophila* and *P. aeruginosa* as test microorganisms because of their well known pathogenicity to invertebrate animals [3]. Cu^{++} as $CuSO_4$ was chosen as the toxicant because of its well documented environmental toxicity and because it has been used extensively in experiments performed by the EERG at UNT.

CHAPTER II

LITERATURE REVIEW

Earthworm Immune System

The earthworm's immune system is located within the coelomic cavity, which contains coelomic fluid and coelomocytes. Coelomocytes are derived from the epithelial lining of the coelomic cavity [4]. Coelomocytes are suspended in the coelomic fluid, a viscous and milky white appearing substance that contains soluble immune factors. Coelomic fluid freely travels between proximal and distal segments of the body through perforations in the septa [5]. Hostetter and Cooper considered the earthworm coelomic cavity to be a precursor to the vertebrate lymphatic system [6].

Coelomocytes are, like mammalian leukocytes, sensitive to foreign materials. They are active in (1) innate immune reactions such as lysozyme production [7], (2) nonspecific immune reactions such as phagocytosis and inflammation, (3) the more complex cellular immune responses responsible for such reactions as graft rejection, and (4) humoral immune responses including synthesis and secretion of agglutinins and lytic factors.

Earthworms possess efficient nonspecific mechanisms of disposing of foreign material, equivalent to the responses found in vertebrates. Earthworm coelomocytes search out, phagocytose, and destroy non-self material [8]. Additionally, coelomocytes mount a

well-defined generalized inflammation in response to tissue injury as part of tissue repair [9-10].

Earthworm coelomocytes synthesize and secrete an array of humoral factors that participate in immune responses. The most important humoral factors are agglutinins and lysins. Agglutinins are specifically induced by and react with antigen [11]. Agglutinins function to aggregate foreign materials and may serve as opsonins providing an efficient mechanism for phagocytosis of foreign organisms such as bacteria, fungi, and viruses [12]. Presence and strength of agglutinins in coelomic fluid can be determined in the same manner as vertebrate antibody and is reported as a titer. Coelomocytes synthesizing and secreting agglutinins are assayed by determining the ability of these cells to form secretory rosettes (SR) with erythrocytes (antigens). SR are formed by coelomocytes releasing agglutinins in response to antigen stimulation, in this case red blood cells, that result in multiple layers of erythrocytes adhering to the coelomocyte [13-15]. Lytic factors inhibit growth of bacteria and thus are important in earthworm defense against pathogens [16-17]. Bacteriostatic and bacteriocidal effects can be induced by inoculating earthworms with sublethal numbers of bacteria, resulting in the immunization of animals and increased resistance to secondary challenge [13].

Cell-mediated immune responses have been demonstrated in earthworms through transplantation studies. Transplantation experiments have demonstrated that earthworms are capable of recognizing and rejecting foreign tissue grafts while accepting autografts [18-19]. Xenografts are rejected more vigorously than allografts. Rejection of a second

transplanted graft from the same donor is accelerated, suggesting a memory component [20-21].

Earthworm Immunological Response to Bacteria

The earthworm immune response to bacteria has been well studied. Coelomic fluid contains natural agglutinins that have been shown to agglutinate bacteria [22], suggesting that they have an antibacterial immune function. Induction of agglutinins has been demonstrated in *Lumbricus* by injecting foreign materials such as vertebrate erythrocytes intracoelomically [23].

Induced agglutinins may result from synthesis, secretion, or the activation of preformed materials, but when compared to vertebrate antibody, they are produced more rapidly [11].

Lumbricus have been shown to contain agglutinins against both Gram-positive and Gram-negative bacteria [24]. These agglutinins, while cross-reactive against a wide variety of bacteria, demonstrate some degree of specific activity. Stein et al. [24] demonstrated that bacterial challenge resulted in agglutinin production with the greatest levels of induced agglutinins specific to the type of bacterium used as the inducing agent. Agglutinins, in addition to aggregating bacteria, also may serve as opsonins providing an efficient mechanism for their phagocytosis. Although hemagglutinin studies have indicated that *Lumbricus* coelomic fluid contains three or four different agglutinin proteins, bacterial absorption analyses suggest that there may be additional immune products that react with microorganisms [24].

In addition to agglutinins, earthworm coelomic fluid also contains lytic factors. Lytic factors have been detected in coelomic fluids collected from both *Eisenia foetida* and *L. terrestris* [25]. Immunoelectrophoresis of *E. foetida* coelomic fluid demonstrated the lytic factor as one of five possible protein components [22]. Lytic factor has been defined

as a lipoprotein that has an activity that can be inhibited by 15 min heating at 56°C [22]. Lytic activity is not inhibited by zymosan, insulin or lipoploysaccharide, nor by hydrazine or methylamine, suggesting that earthworm hemolysins are not related to C3 or C3b complement components [26]. Use of a modified Jerne's plaque assay, indirect fluoresence, transmission electron microscopy with peroxidase labeling, and scanning electron microscopy have demonstrated that chloragogen cells, rich in endoplasmic reticulum and basophilic coelomocytes, synthesize and release lytic factors [27].

Lytic factor molecules possess bacteriostatic activity against bacteria shown to be pathogenic for earthworms (e.g. *Bacillus thuringiensis, Bacillus megaterium,* and *A. hydrophila*) [28]. Because of the strong bacteriostatic activity of coelomic fluid in *E. foetida*, the lysin has been named *Eisenia foetida andrei* Factor (EFAF) [22]. The activity of EFAF seems important in earthworm defense against potential pathogens. Lassegues et al. [17] reported that injection of *A. hydrophila* and *B. megaterium* resulted in an enhancement of antibacterial activity, however lytic activity was not specific for either of the two pathogenic bacteria.

A lysozyme like enzyme has been identified and purified from the *Eisenia* coelomic fluid [29] and identified in *L. terrestris* coelomic fluid [7]. Lysozyme, one of the best known antimicrobial factors, is a bacteriolytic enzyme for Gram-positive bacteria, specifically directed against the cell wall mucopeptide N-acetyl muramic acid-N-acetyl glucosamine (NAM-NAG). Lysozyme is a basic low molecular weight monomeric protein with intrachain disulfide bonds; its lytic activity is stable when heated in acidic medium and disappears when heated in an alkaline one [30]. Studies of Cotuk and Dales in 1984 [29]

and Kauschke and Mohrig [31] in 1987 showed that activity of the coelomocyte extracts agree with the criteria for a lysozyme, but the normal level in coelomic fluid is very low. However, Lassalle et al. [30] reported the existence of active and significant lysozyme-like activity against *Micrococcus lysodeikticus* in *E. foetida andrei* coelomic fluid using basically the same method. Lassalle et al. [30] isolated the active protein by fast protein liquid chromatography and characterized it as lysozyme because of its activity against *M. lysodeikticus* cell wall, heat stability at acidic pH, low lability at basic pH and molecular weight of approximately 20,000 daltons. Goven et al. [7] demonstrated that lysozyme present in *L. terrestris* coelomocyte extracts and coelomic fluid showed similar activity to that of lysozyme collected from human and murine leukocyte extracts.

Earthworm Model for Immunotoxicity

In 1988 the EERG began developing a model immunoassay system using earthworms, *Lumbricus terrestris*, as nonvertebrate surrogates for assessing immunotoxic potential of chemicals and understanding their modes of action. Development of an earthworm immune-based system of biomarkers was based on a need for rapid, sensitive, cost-effective, and socially non-controversial surrogate immunoassay protocols that could be used as an adjunct or complement to existing protocols with mammals. Such an assay system would be used to screen chemicals to determine if further mammalian tests should be performed.

Earthworms were selected for several reasons: (1) Their immune functions appear to be sufficiently analogous or homologous to those in vertebrates for use in screening

chemicals for immunotoxicity in higher organisms, including mammals, (2) being virtually ubiquitous and ecologically important soil organisms, they are valuable *in situ* sentinels for use in assessing risks to public and environmental health, and (3) earthworm behavior and morphology enable their direct exposure to complex environmental mixtures and matrices of pollutants. Additionally, earthworms are easy and inexpensive to maintain and conduct immunological experiments with, their basic biology is well known, and they are currently used in standardized acute toxicity protocols for laboratory and *in situ* bioassays.

CHAPTER III

MATERIALS AND METHODS

Source and Maintenance of Earthworms

Earthworms were obtained commercially from Carolina Biological Supply (Burlington, NC, USA). Stock *L. terrestris* were maintained at 10 ± 2^{o}C in plastic containers (70 x 40 x 15 cm) in continuous darkness in media composed of four parts potting soil to one part peat moss, moistened with doubled distilled, deionized water. All earthworms were held in this environment for a minimum of three days prior to use. Worms were fed commercial dry high protein baby cereal (Gerber, Fremont, MI, USA), replenished as needed. Unused earthworms were transferred to a new soil mixture every two to three weeks.

Source and Maintenance of Microorganisms

A. hydrophila (ATCC 19570) was purchased from American Type Culture Collection (Rockville, MD, USA). *P. aeruginosa* was obtained from the UNT stock culture collection. Bacterial cultures were maintained on Tryptic Soy Agar (TSA) (Difco Laboratories, Detroit, MI, USA) slants at 30^{o}C until use.

Culture Conditions and Standardization of Bacterial Suspensions

For working cultures, cells from either *A. hydrophila* or *P. aeruginosa* slants were inoculated into test tubes containing five ml of Tryptic Soy Broth (TSB) (Difco

Laboratories, Fremont, MI, USA) after which the cultures were incubated at 28^{o}C for 24 h on a shaker agitated at 100 rpm. These initial cultures were used to inoculate a 250 ml flask containing 50 ml of TSB. These cultures were incubated as described above.

Cell numbers were determined by the spread plate method of serial 10-fold dilutions. For each dilution dual plates of TSA were spread with a 0.1 ml suspension and incubated at 28°C for 18-24 h, after which colonies were counted and the mean number of bacteria determined as colony forming units (CFU). CFU were correlated with the A_{620} of the original suspension. Standard suspensions were prepared by diluting cultures to the appropriate A to give the cell concentration desired. For experiments, bacterial suspensions (10 ml) were washed once in Hanks Balanced Salt Solution (HBSS) (Sigma, St. Louis, MO, USA) by centrifuging at 3200 x g for 10 min and then resuspending with HBSS to the desired concentration.

Determination of Earthworm Resistance to Oral and Intracoelomic Bacterial Inoculation

For injection experiments *A. hydrophila* and *P. aeruginosa* cultures were adjusted to concentrations of 10^5, 10^6, 10^7, 10^8, and 10^9 CFU/ml in TSB. Before injecting bacteria, worms were washed in ice cold 0.85% saline. Using a sterile syringe fitted with a 27 1/2 gauge needle, a total volume of 0.1 ml of the appropriate bacterial suspension was inoculated into the coelomic cavity, making several separate punctures into various body segments posterior to the clitellum. Ten worms were inoculated with each concentration yielding earthworms injected with final cell numbers of 10^4, 10^5, 10^6, 10^7,

and 10^8 of the appropriate organism. Ten worms were inoculated with 0.1 ml of TSB and served as a control group.

After intracoelomic inoculation the worms were placed in 12.5 cm by 7.5 cm ziplock bags (Zip-Pak, Seguin, TX, USA) containing 90 cm^2 moistened Whatman #2 filter paper (Whatman, Maidstone, England). Earthworms were housed in complete darkness at 10°C and checked daily for 14 days to determine mortality. Mortality was determined by observing the earthworm's response to physical stimulation to its anterior end.

For oral exposure experiments, bacteria and earthworms were prepared as described above. Oral bacterial inoculation was accomplished using an intubation needle. Intubation was accomplished by applying gentle pressure to the oral cavity, resulting in the earthworm accepting the rounded end of the needle into its oral cavity. Ten worms were inoculated with each concentration yielding earthworms intubated with final cell numbers of 10^4 , 10^5, 10^6, 10^7, and 10^8 of the appropriate organism. Ten worms were inoculated with 0.1 ml of TSB and served as a control group.

Following oral intubation earthworms were treated as described above. Mortality was determined in the same manner as described for earthworms that were injected.

Copper Lethal Exposure

Prior to Cu^{++} exposure, earthworms were washed in ice cold 0.85% saline. The method of Giggleman [32] was used to expose earthworms. Individuals were exposed

using 90 cm^2 Whatman #2 filter paper rectangles within 1.2 x 1.97 cm zip-lock bags. Filter papers were saturated with 1.5 ml of eight different Cu^{++} concentrations (0.03, 0.06. 0.12, 0.18, 0.24, 0.36, 0.48, 0.6 mg Cu^{++}/ml) that resulted in nominal exposure concentrations of 0.5,1.0, 2.0, 3.0, 4.0, 6.0, 8.0, and 10.0 μg/cm^2 Cu^{++}, respectively. Solutions were prepared by dissolving $CuSO_4$ (98% purity, MCB, Norwood, OH, USA) in double-distilled, deionized water. Controls were exposed to filter paper saturated only with double-distilled, deionized water. Each exposure concentration group, consisting of 10 earthworms, was held for 120 h in the dark at 10 ± 2°C within an environmental chamber. Worms were checked daily. Nominal filter paper concentrations and mortality data were used to calculate lethal concentrations, LC_{50}s.

Exposure of Earthworms to Copper and Bacteria

To determine the effect of Cu^{++} exposure on earthworm resistance to bacterial infection, earthworms were exposed to sublethal Cu^{++} concentrations of 1.0, 2.0, and 3.0 μg/cm^2 for 120 h on filter paper as previously described. Immediately following Cu^{++} exposure earthworms were inoculated orally or intra-coelomically with $1x10^8$ *A. hydrophila* or *P. aeruginosa* organisms as indicated above. Control groups consisted of earthworms exposed to filter paper saturated with double distilled deionized water and no Cu^{++} or bacteria, arthworms exposed to 3 μg/cm^2 Cu^{++} and no bacteria, and earthworms exposed orally or intracoelomically to the appropriate bacterial species but not exposed to Cu^{++}. Each experimental and control group consisted of 10 earthworms.

Collection of Coelomic Fluid and Agglutination Determination

To determine if Cu^{++} affects the ability of coelomic fluid soluable factors to aggregate particulate matter as part of an antibacterial response, agglutination studies using rabbit red blood cells (RRBC) (Cleveland Scientific, Cleveland, OH, USA) were performed. For these experiments coelomic fluid was collected from individual earthworms by inserting a sharpened Pasteur pipette into the coelom posterior to the clitellum and allowing it to fill by intra-coelomic pressure [33]. Coelomic fluid was pooled from six worms and transferred to a microcentrifuge tube and centrifuged at 500 x g for 10 min to yield a cell-free supernatant.

Coelomic fluid agglutination activity was titrated by making doubling serial dilutions using Hanks Balanced Salt Solution (HBSS) (Sigma Chemical Co., St. Louis, MO, USA) from 1:2 (25 µl coelomic fluid:25 µl HBSS) to 1:4096 in 200 µl microtiter plate wells. To test for agglutination activity 25 µl of a 2% suspension of RRBCs in HBSS was added to each well. Microtiter plates were incubated for 24 h at 10°C. Agglutination titer was determined using a Dynatech Mictotiter MR 7000 Plate Reader (Dynatech Laboratories, Chantilly, VA, USA). The titer was defined as the greatest dilution of coelomic fluid demonstrating the desired effect, aggregation of RRBCs.

Chemical Analysis of Earthworm Tissue

At the conclusion of each experiment worms were killed and the gut removed. Worm carcasses were frozen at $^{-}4^{o}C$. Prior to tissue digestion each worm was dried at $50^{o}C$ in an acid-rinsed, pre-weighed 15 ml beaker for 24-h. Following drying the dry

weight of each worm was determined. For tissue processing two ml of double distilled, deionized water were added to each sample and tissue was shredded with a cell disrupter sonifier (Tissue-Tearor^{TM}, # 985-370, Biospec Products, Bartlesville, OK, USA) until the mixture was homogeneous. When $CuSO_4$ "spiking" was desired, 10 µg $CuSO_4$ was added to the 2 ml of double distilled, deionized water. Between samples the sonifier was cleaned with a (1:1, v/v) solution of hexane (99.05 purity, Optima Grade, Fisher, Fair Lawn, NJ, USA) and acetone (Fisher, Fair Lawn, NJ, USA). Following homogenization, each beaker was covered with a watch glass and samples were digested with eight ml of 60% HNO_3 in double distilled, deionized water for 2.5-h on a hot plate at 50°C. Caution was taken to insure only slight bubbling so each sample would have a final volume of ten ml. Samples were stored at room temperature in 12 ml glass tubes with Teflon-lined screw caps until analysis was complete. Cu^{++} content was examined by atomic absorption spectrometry at TRAC Laboratories, Inc. (Denton, TX, USA) using methods published by the USEPA [34].

Statistical Analysis

Data were tested for normality, homogeneity and statistical significance using guidelines published by Zar [35]. Statistical tests were run using software published by the SAS Institute [36].

CHAPTER IV

RESULTS

Copper Lethal Exposure

Exposure resulted in a 120 h-LC_{50} of 6.16 µg/cm^2 (95% Confidence Interval: 5.18 - 7.24) for *L. terrestris* (Table 1, Figure 1). Exposure of earthworms to concentrations of 3.0 µg Cu^{++}/cm^2 and below was completely sublethal and only one animal died at 4.0 µg Cu^{++}/cm^2 exposure. Lethality of Cu^{++} increased with exposure concentrations greater than 4.0 µg Cu^{++}/cm^2 with 100% mortality at 10.0 µg Cu^{++}/cm^2 occurring after 5-d exposure.

Bacterial Pathogenicity

Pathogenicity of *A. hydrophila* and *P. aeruginosa* at doses ranging from 10^4-10^8 CFU/worm, administered orally or intracoelomically, was determined by measuring mortality produced in earthworms over a 14-d period post-infection (Tables 2-5, Figure 2). Neither of these organisms was found to be very pathogenic at the bacterial concentrations used or routes administered.

Mortality of *L. terrestris* injected with *A. hydrophila* was first observed five days following injection of bacteria into the coelomic cavity, with 10% mortality found in

Table 1. Relation of mortality in earthworms, *Lumbricus terrestris*, to 120-h filter paper exposure to Cu^{++} ($CuSO_4$).

Nominal Exposure Concentration ($\mu g/cm^2$)	Daily mortality: dead/live (% mortality)*				
	Day 1	Day 2	Day 3	Day 4	Day 5
10.0	1/9(10)	2/8(20)	2/8(20)	5/5(50)	10/0(100)
8.0	0/10(0)	1/9(10)	2/8(20)	3/7(30)	6/4(60)
6.0	0/10(0)	0/10(0)	0/10(0)	3/7(30)	6/4(60)
4.0	0/10(0)	0/10(0)	0/10(0)	0/10(0)	1/9(10)
3.0	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)
2.0	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)
1.0	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)
0.5	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)
Controls	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)

LC_{50} = 6.16 $\mu g/cm^2$ 95% CL: 5.18-7.24

* Each group consisted of 10 earthworms.

Figure 1. Mortality in earthworms, *Lumbricus terrestris,* following a 120-h filter paper exposure to Cu^{++} as $CuSO_4$. LC_{50} = 6.16 μg/cm^2

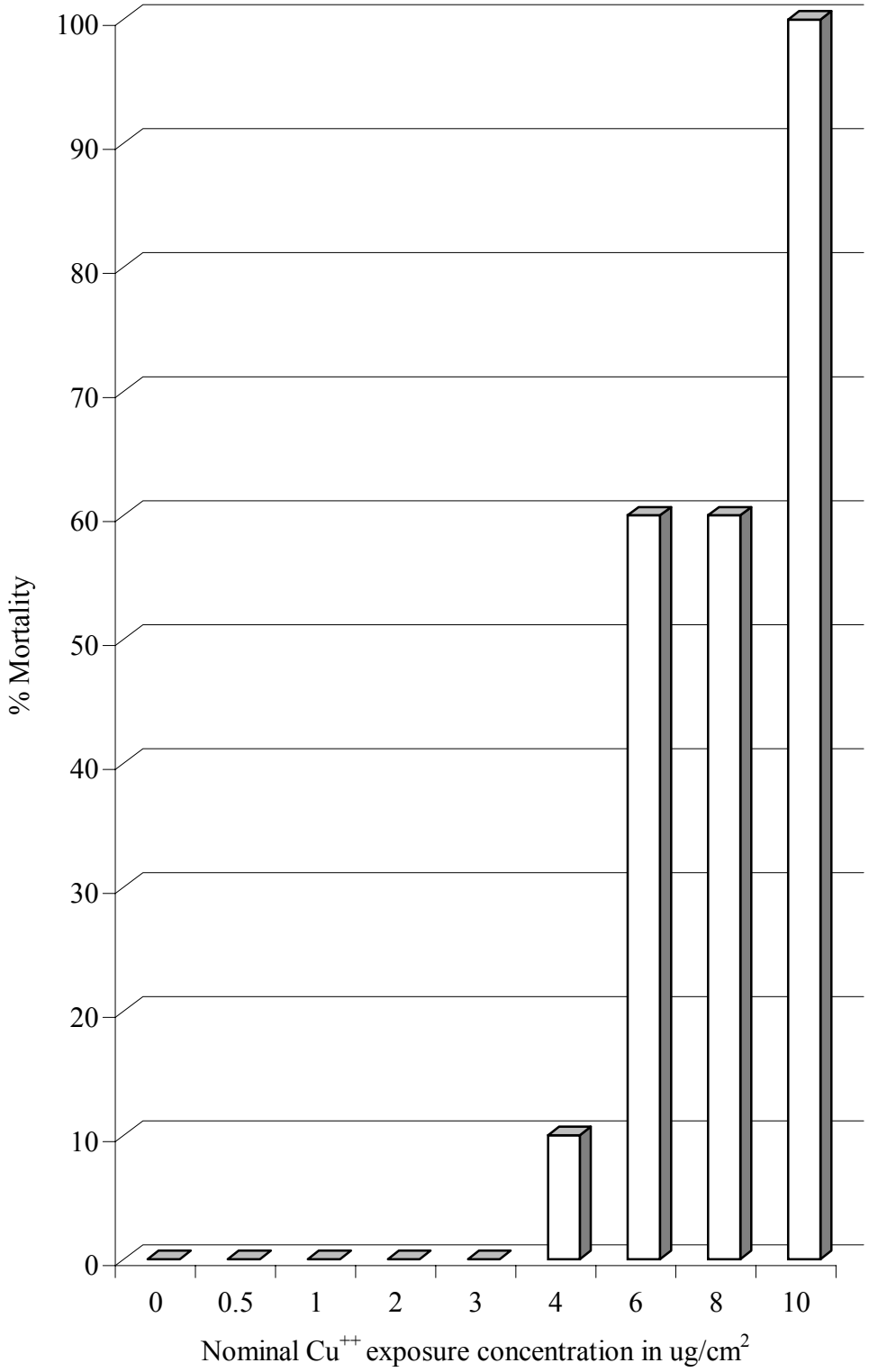

100
90
80
70
60
50
40
30
20
10
0
% Mortality
0
0.5
1
2
3
4
6
8
10
Nominal Cu++ exposure concentration in ug/cm2

Table 2. Relation of mortality in earthworms, *Lumbricus terrestris*, to intracoelomic injection of *Aeromonas hydrophila* quantified as colony forming units (CFU).

	Daily mortality: dead/live (% mortality)					
		CFU				
Days Post-Injection	Controls	$1x10^4$	$1x10^5$	$1x10^6$	$1x10^7$	$1x10^8$
1	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)
2	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)
3	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)
4	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)
5	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)	1/9(10)
6	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)	1/9(10)
7	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)	1/9(10)
8	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)	1/9(10)
9	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)	1/9(10)
10	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)	1/9(10)
11	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)	1/9(10)
12	0/10(0)	0/10(0)	1/9(10)	0/10(0)	0/10(0)	1/9(10)
13	0/10(0)	0/10(0)	1/9(10)	0/10(0)	0/10(0)	1/9(10)
14	0/10(0)	1/9(10)	1/9(10)	0/10(0)	1/9(10)	1/9(10)

Table 3. Relation of mortality in earthworms, *Lumbricus terrestris*, to oral intubation of A*eromonas hydrophila* quantified as colony forming units (CFU).

	Daily mortality: dead/live (% mortality)					
		CFU				
Days Post-Intubation	Controls	$1x10^4$	$1x10^5$	$1x10^6$	$1x10^7$	$1x10^8$
1	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)
2	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)
3	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)
4	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)
5	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)
6	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)
7	0/10(0)	1/9(10)	1/9(10)	0/10(0)	1/9(10)	1/9(10)
8	0/10(0)	1/9(10)	1/9(10)	0/10(0)	1/9(10)	1/9(10)
9	0/10(0)	1/9(10)	1/9(10)	0/10(0)	1/9(10)	1/9(10)
10	0/10(0)	2/8(20)	1/9(10)	0/10(0)	1/9(10)	1/9(10)
11	0/10(0)	2/8(20)	1/9(10)	0/10(0)	1/9(10)	1/9(10)
12	0/10(0)	2/8(20)	1/9(10)	0/10(0)	1/9(10)	1/9(10)
13	0/10(0)	2/8(20)	1/9(10)	1/9(10)	1/9(10)	1/9(10)
14	0/10(0)	2/8(20)	1/9(10)	1/9(10)	1/9(10)	1/9(10)

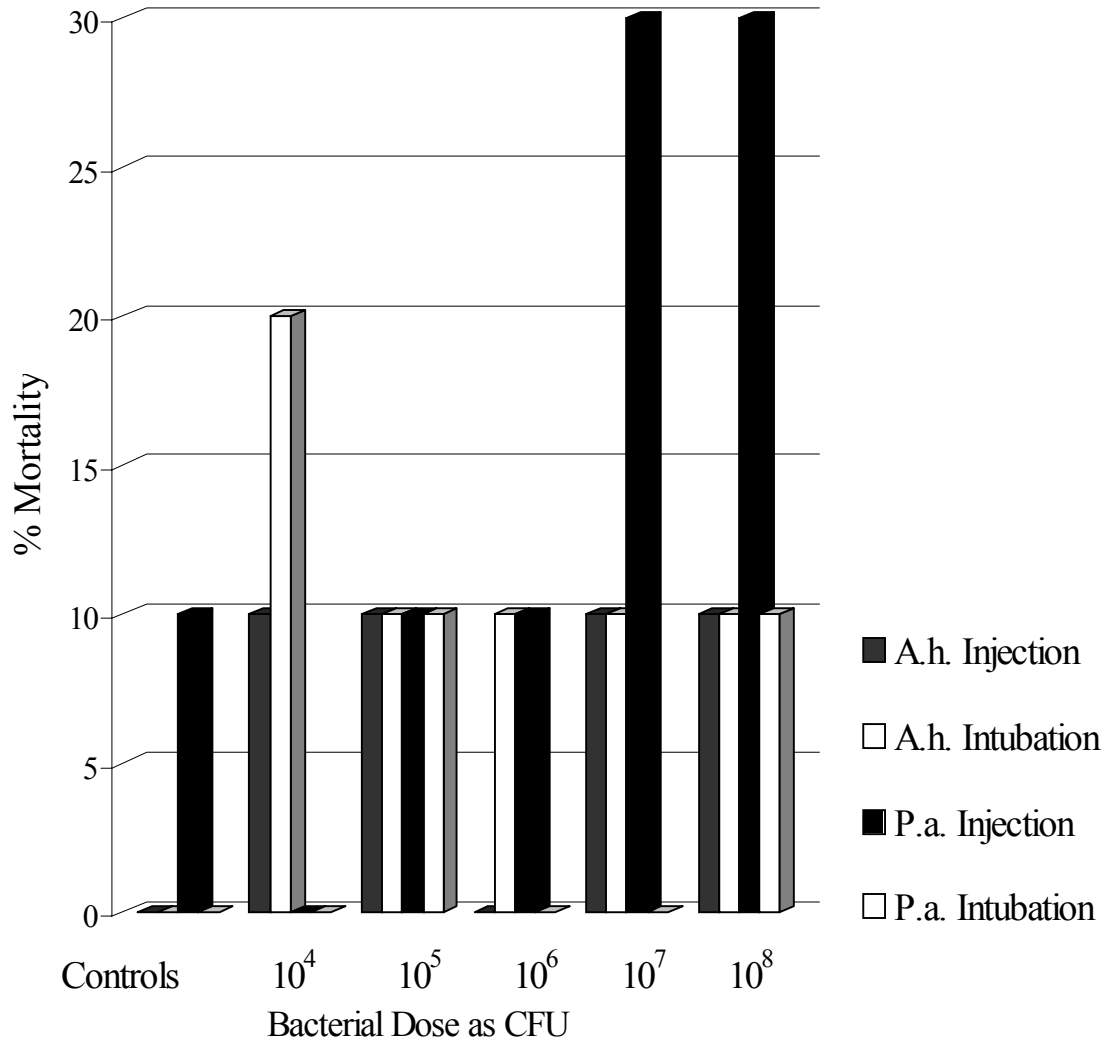
Table 4. Relation of mortality in earthworms, *Lumbricus terrestris*, to intracoelomic injection of *Pseudomonas aeruginosa* quantified as colony forming units (CFU).

	Daily mortality: dead/live (% mortality)					
		CFU				
Days Post-Injection	Controls	$1x10^4$	$1x10^5$	$1x10^6$	$1x10^7$	$1x10^8$
1	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)
2	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)
3	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)
4	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)
5	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)
6	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)
7	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)
8	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)
9	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)
10	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)
11	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)
12	0/10(0)	0/10(0)	1/9(10)	0/10(0)	0/10(0)	3/7(30)
13	1/9(10)	0/10(0)	1/9(10)	1/9(10)	3/7(30)	3/7(30)
14	1/9 (10)	0/10(0)	1/9(10)	1/9(10)	3/7(30)	3/7(30)

Table 5. Relation of mortality in earthworms, *Lumbricus terrestris*, to oral intubation of *Pseudomonas aeruginosa* quantified as colony forming units (CFU).

	Daily mortality: dead/live (% mortality)					
		CFU				
Days Post-Intubation	Controls	$1x10^4$	$1x10^5$	$1x10^6$	$1x10^7$	$1x10^8$
1	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)
2	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)
3	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)
4	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)
5	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)
6	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)
7	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)
8	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)
9	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)
10	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)
11	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)
12	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)
13	0/10(0)	0/10(0)	1/9(10)	0/10(0)	0/10(0)	1/9(10)
14	0/10(0)	0/10(0)	1/9(10)	0/10(0)	0/10(0)	1/9(10)

Figure 2. Mortality in earthworms, *Lumbricus terrestris*, 14-d following oral intubation or intracoelomic injection of *Aeromonas hydrophila* (*A. h*.) or *Pseudomonas aeruginosa* (*P. a*.) quantified as colony forming units (CFU).


30
25
20
15
10
5
0
% Mortality
Controls
10^4
10^5
10^6
10^7
10^8
Bacterial Dose as CFU
A.h. Injection
A.h. Intubation
P.a. Injection
P.a. Intubation

earthworms injected with 10^8 CFU. On Day 12, 10% mortality was observed in earthworms injected with 10^5 CFU. On Day 14, a 10% mortality was observed in worms injected with 10^4 and 10^7 CFU. No other mortality was observed among earthworms in this experiment, including those serving as controls (Table 2). Mortality of *L. terrestris* administered an oral dose of *A. hydrophila* was first observed on Day 7 with a 10% mortality found in earthworms given 10^4,10^5, 10^7, and10^8 CFU. On Day 10, 20% mortality was observed in animals given 10^4 CFU. A 10% mortality was observed by Day 13 in earthworms given 10^6 CFU . All control worms lived throughout this experiment (Table 3).

Mortality among *L. terrestris* injected with *P. aeruginosa* was first observed on Day 12 post-intracoelomic injection with a 10% mortality in 10^5 CFU and 30% mortality in 10^8 CFU inoculated worms. On Day 13, a 10% mortality was demonstrated in earthworms given 10^6 CFU, while a 30% mortality was found in those given 10^7 CFU. Control earthworms demonstrated a 10% mortality. No other earthworms died (Table 4). Mortality of worms given oral doses of *P. aeruginosa* was only observed near the end of the experiment,on Day 13, with a 10% mortality for worms dosed with 10^5 and 10^8 CFU. All control worms lived throughout the experiment (Table 5).

Figure 2 summarizes Tables 2-5 by demonstrating the percent cumulative mortality on Day 14, the final day of the experiment, as a function of bacterial dose. The highest mortality, 30%, occurred in worms injected with 10^7 or 10^8 *P. aeruginosa* CFU.

Exposure of Earthworms to Copper and Bacteria

In general, exposure to Cu^{++} did not lower the resistance of earthworms to bacterial challenge. Only oral dosing of *A. hydrophila* resulted in lower resistance following Cu^{++} exposure as compared to bacterial challenge alone. Greater or equal resistance was observed in all injected worms versus those getting oral doses. The highest mortality rate was 40%, observed in worms exposed for 120-h to 3 μg Cu^{++}/cm^2 prior to P. aeruginosa injection (Figure 3). Mortality rates reported in Tables 1-9 and Figures 2-3 are not significantly different from controls when compared using the two tailed, unpaired t test, as all p are > 0.05.

Mortality of *L. terrestris* administered an injection of *A. hydrophila* following a 120-h Cu^{++} exposure was first observed on Day 2 post-injection, when 10% of worms exposed to 2 and 3 μg Cu^{++}/cm^2 died. On Day 3, a cumulative 20% mortality was found in worms exposed to 2 μg Cu^{++}/cm^2. On Day 9, 10% of worms exposed to 1 μg Cu^{++}/cm^2 died. On Day 10, mortality of worms exposed to 3 μg Cu^{++}/cm^2 reached 20%. This increased to 30% mortality for worms exposed to both 2 and 3 μg Cu^{++}/cm^2 by Day 11. In the control groups one worm died in the group in which worms were exposed to Cu^{++} only (Table 6).

Mortality of worms administered an oral dose of *A. hydrophila* following a 120-h Cu^{++} exposure was limited to 10% of worms exposed to 2 μg Cu^{++}/cm^2 on Day 13 (Table 7).

Mortality of *L. terrestris* injected with *P. aeruginosa* following a 120-h Cu^{++} exposure was first observed on Day 2 post-injection, when 10% of worms exposed to 3 µg Cu^{++}/cm^2 died. On Day 6, 10% of worms exposed to 2 µg Cu^{++}/cm^2 were found to be dead. On Day 8, mortality of worms exposed to 3 µg Cu^{++}/cm^2 increased to 20%. Mortality of worms exposed to 3 µg Cu^{++}/cm^2 increased to 30% on Day 11 and 40% on Day 13. On Day 8 a 10% mortality was found in worms exposed to Cu^{++} only (Table 8).

Mortality of worms administered an oral dose of *P. aeruginosa* following a 120-h exposure to Cu^{++} was first observed on Day 10, with 10% mortality of *L. terrestris* exposed to 3 µg Cu^{++}/cm^2. Additional mortality occurred on Day 13, when 10% of worms exposed to 2 µg Cu^{++}/cm^2 died (Table 9).

Table 6. Relation of mortality in earthworms, *Lumbricus terrestris*, to intracoelomic injection of *Aeromonas hydrophila* quantified as colony forming units (CFU) following a 120-h exposure to Cu^{++} as $CuSO_4$.

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	Daily mortality: dead/live (% mortality)					
Days Post-Injection	3 µg/cm^2 1x10^8 CFU	2 µg/cm^2 1x10^8 CFU	1 µg/cm^2 1x10^8 CFU	0 Cu^{++} 0 CFU	3 µg/cm^2 Cu^{++} 0 CFU	0 Cu^{++} 1x10^8 CFU
1	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)
2	1/9(10)	1/9(10)	0/10(0)	0/10(0)	0/10(0)	0/10(0)
3	1/9(10)	2/8(20)	0/10(0)	0/10(0)	0/10(0)	0/10(0)
4	1/9(10)	2/8(20)	0/10(0)	0/10(0)	0/10(0)	0/10(0)
5	1/9(10)	2/8(20)	0/10(0)	0/10(0)	0/10(0)	0/10(0)
6	1/9(10)	2/8(20)	0/10(0)	0/10(0)	0/10(0)	0/10(0)
7	1/9(10)	2/8(20)	0/10(0)	0/10(0)	1/9(10)	0/10(0)
8	1/9(10)	2/8(20)	0/10(0)	0/10(0)	1/9(10)	0/10(0)
9	1/9(10)	2/8(20)	1/9(10)	0/10(0)	1/9(10)	0/10(0)
10	2/8(20)	2/8(20)	1/9(10)	0/10(0)	1/9(10)	0/10(0)
11	3/7(30)	3/7(30)	1/9(10)	0/10(0)	1/9(10)	0/10(0)
12	3/7(30)	3/7(30)	1/9(10)	0/10(0)	1/9(10)	0/10(0)
13	3/7(30)	3/7(30)	1/9(10)	0/10(0)	1/9(10)	0/10(0)
14	3/7(30)	3/7(30)	1/9(10)	0/10(0)	1/9(10)	0/10(0)

Table 7. Relation of mortality in earthworms, *Lumbricus terrestris,* to oral intubation of *Aeromonas hydrophila* quantified as colony forming units (CFU) following a 120-h exposure to Cu^{++} as $CuSO_4$.

	Daily mortality: dead/live (% mortality)					
Days Post-Intubation	3 μg/cm^2 1x10^8 CFU	2 μg/cm^2 1x10^8 CFU	1 μg/cm^2 1x10^8 CFU	0 Cu^{++} 0 CFU	3 μg/cm^2 Cu^{++} 0 CFU	0 Cu^{++} 1x10^8 CFU
1	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)
2	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)
3	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)
4	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)
5	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)
6	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)
7	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)
8	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)
9	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)
10	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)
11	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)
12	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)
13	0/10(0)	1/9(10)	0/10(0)	0/10(0)	0/10(0)	0/10(0)
14	0/10(0)	1/9(10)	0/10(0)	0/10(0)	0/10(0)	0/10(0)

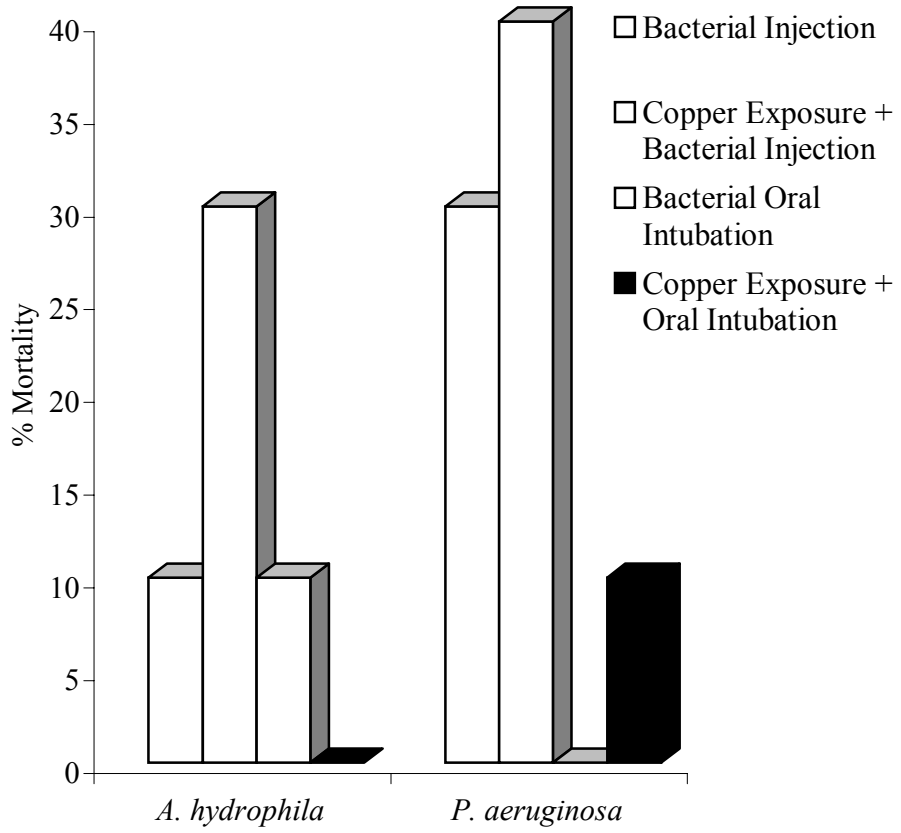
Table 8. Relation of mortality in earthworms, *Lumbricus terrestris*, to intracoelomic injection of *Pseudomonas aeruginosa* quantified as colony forming units (CFU) following a 120-h exposure to Cu^{++} as $CuSO_4$.

	Daily mortality: dead/live (% mortality)					
Days Post-Injection	3 µg/cm^2 1x10^8 CFU	2 µg/cm^2 1x10^8 CFU	1 µg/cm^2 1x10^8 CFU	0 Cu^{++} 0 CFU	3 µg/cm^2 Cu^{++} 0 CFU	0 Cu^{++} 1x10^8 CFU
1	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)
2	1/9(10)	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)
3	1/9(10)	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)
4	1/9(10)	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)
5	1/9(10)	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)
6	1/9(10)	1/9(10)	0/10(0)	0/10(0)	0/10(0)	0/10(0)
7	1/9(10)	1/9(10)	0/10(0)	0/10(0)	0/10(0)	0/10(0)
8	2/8(20)	1/9(10)	0/10(0)	0/10(0)	1/9(10)	0/10(0)
9	2/8(20)	1/9(10)	0/10(0)	0/10(0)	1/9(10)	0/10(0)
10	2/8(20)	1/9(10)	0/10(0)	0/10(0)	1/9(10)	0/10(0)
11	3/7(30)	1/9(10)	0/10(0)	0/10(0)	1/9(10)	0/10(0)
12	3/7(30)	1/9(10)	0/10(0)	0/10(0)	1/9(10)	0/10(0)
13	4/6(40)	1/9(10)	0/10(0)	0/10(0)	1/9(10)	0/10(0)
14	4/6(40)	1/9(10)	0/10(0)	0/10(0)	1/9(10)	0/10(0)

Table 9. Relation of mortality in earthworms, *Lumbricus terrestris*, to oral intubation of *Pseudomonas aeruginosa* quantified as colony forming units (CFU) following a 120-h exposure to Cu^{++} as $CuSO_4$.

	Daily mortality: dead/live (%mortality)					
Days Post-Intubation	3 µg/cm^2 1x10^8 CFU	2 µg/cm^2 1x10^8 CFU	1 µg/cm^2 1x10^8 CFU	0 Cu^{++} 0 CFU	3 µg/cm^2 Cu^{++} 0 CFU	0 Cu^{++} 1x10^8 CFU
1	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)
2	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)
3	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)
4	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)
5	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)
6	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)
7	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)
8	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)
9	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)
10	1/9(10)	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)
11	1/9(10)	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)
12	1/9(10)	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)
13	1/9(10)	1/9(10)	0/10(0)	0/10(0)	0/10(0)	0/10(0)
14	1/9(10)	1/9(10)	0/10(0)	0/10(0)	0/10(0)	0/10(0)

Figure 3. Cumulative 14-d mortality in earthworms, *Lumbricis terrestris*, exposed to 3 μg Cu^{++}/cm^2 for 120-h prior to oral intubation or intracoelomic injection of $1x10^8$ colony forming units of *Aeromonas hydrophila* or *Pseudomonas aeruginosa* as compared to earthworms only exposed to bacterial challenge.



Effect of Copper Exposure on Agglutination of Rabbit Red Blood Cells by Earthworm Coelomic Fluid

The distributions of agglutination titers found for coelomic fluid collected from Cu^{++}-exposed and distilled water-exposed worms were significantly different than normal, $p < 0.05$. Ranked titers were significantly different between coelomic fluid collected from worms treated with Cu^{++} and those exposed to distilled water as determined by Tukey-like nonparametric multiple range test. Median titers were 320.0 and 40.0 for Cu^{++} and water exposed worms, respectively (Table 10).

Tissue Concentration of Copper

Tissue concentrations of Cu^{++} were determined in earthworms immediately after 120-h filter paper exposure to 3 μg Cu^{++}/cm^2 (Table 11) The Cu^{++} content of the worms exposed to 3 μg Cu^{++}/cm^2 was significantly greater than that found in control worms exposed to distilled water, Student-Newman-Keuls test, $\alpha = 0.05$ (Table 11). Means for Cu^{++} and water exposed worms were 35.70 ± 12.08 μg/g and 5.31 ± 1.67 μg/g, respectively.

Table 10. Ability of coelomic fluid collected from earthworms, *Lumbricus terrestris*, after 120-h filter paper exposure to 3.0 μg/cm^2 Cu^{++} as $CuSO_4$ to agglutinate rabbit red blood cells.

Cu^{++} Exposed		H_2O Exposed Controls	
	Titer		Titer
Pool*	1 256.0	Pool*	1 256.0
	2 4096.0		2 153.6
	3 4096.0		3 12.0
	4 8192.0		4 51.2
	5 26.0		5 64.0
	6 384.0		6 13.6
	7 115.2		7 28.8
	8 115.2		8 16.0
	9 512.0		Median = 40.0
	10 112.0		
	11 56.0		
	12 512.0		
	Median = 320.0**		

* Pool represents coelomic fluid collected from six earthworms.
** Significantly different from controls, Tukey-like nonparametric multiple range test, $\alpha = 0.05$. Distributions are significantly different than normal, $p<0.05$.

Table 11. Tissue concentration (µg/g dry mass) of copper in earthworms, *Lumbricus terrestris*, exposed to 3 µg/cm^2 Cu^{++} using a 120-h filter paper protocol.

Cu^{++} Exposed		DI H_2O Exposure	
Tissue Concentration		Tissue Concentration	
Pool*	1	Pool*	1
	46.43		5.14
	2		2
	51.70		6.13
	3		3
	34.68		4.06
	4		4
	15.23		5.41
	5		5
	24.94		5.83
	6		
	27.32		
	7	Mean	= 5.31 ± 1.67
	32.44	CV	= 31.5
	8		
	24.69		
	9		
	40.75		
	10		
	41.94		
	11		
	52.60		
	12		
	48.04		

Mean = 35.70 ± 12.08
CV = 33.8

* Pool represents coelomic fluid collected from six earthworms.
Groups are significantly different, Student-Newman-Keuls parametric test, $\alpha = 0.05$.

CHAPTER V
DISCUSSION

In this research I attempted to identify microbial organisms, pathogenic to *L. terrestris*, for use in a biomarker assay designed to predict the immunosuppressive potential of chemicals. I used *A. hydrophila* and *P. aeruginosa* as microbial agents and Cu^{++} as a test chemical. This study was a part of a larger ongoing research project to develop a system of biomarkers to assess the immunotoxicity of chemicals by measuring their effects on host resistance to challenge with pathogenic organisms.

Copper: Lethal and Sublethal Exposures

Cu^{++} toxicity found in this study can be compared with results by Giggleman et al. [32] and Goven et al. [7]. Giggleman et al. [32] filter paper 96-h LC_{50} of 7.6 µg Cu^{++}/cm^2 and 70% mortality of worms exposed to 10 µg Cu^{++}/cm^2 compares favorably to results in this study (120-h LC_{50} = 6.16 µg Cu^{++}/cm^2 and 100% mortality of earthworms exposed to 10 µg Cu^{++}/cm^2). Since I did not report a body-burden based LD_{50}, more direct comparisons of the data are not possible. Goven et al. [7] reported a 120-h LC_{50} of 2.6 µg Cu^{++}/cm^2 and 100% mortality of earthworms exposed to 8 µg Cu^{++}/cm^2. This LC_{50} is approximately 40.0% of my 120-h LC_{50}. Again, since neither Goven et al. [7] nor I reported a body-burden-based LD_{50} , direct comparisons are not possible.

Cu^{++} has also been reported as being toxic to *E. foetida*, also an earthworm. Newhauser et al. [37] and Edwards et al. [38] reported 48-h LC_{50}s of 6.3 μg Cu^{++}/cm^2 and 10.4 μg Cu^{++}/cm^2 , respectively. Based on the Contact Toxicity Test developed by Roberts and Dorough [39], Cu^{++} can be classified as very toxic to *L. terrestris* and extremely toxic *to E. foetida*.

There are few studies available that report on the sublethal immunologic effects of Cu^{++} in earthworms. Studies do indicate that Cu^{++} exposure reduces both the ability of *L. terrestris* coelomocytes to phagocytose microorganisms and kill those once they are ingested. Goven et al. [7] demonstrated that body concentrations of 29 μg Cu^{++}/g dry tissue mass, concentrations lower than those needed to suppress phagocytosis as demonstrated by Giggleman [32] (49 μg Cu^{++}/ g dry tissue mass), reduced lysozyme activity of coelomic fluid and coelomocyte extracts [7]. Additionally, Chen et al. [40] reported that Cu^{++} at a tissue concentration of 10 μg Cu^{++}/g dry mass inhibited the ability of coelomocytes to reduce Nitroblue Tetrazolium (NBT) dye, indicating indirectly that Cu^{++} exposure reduces the ability of these cells to kill ingested microorganisms by oxygen-dependent mechanisms. For these reasons Cu^{++} is believed to interfere with both innate (lysozyme) and non-specific (phagocytosis and oxygen-dependent killing) immunologic defenses found in earthworms that are common to all metazoans.

Pathogenicity Testing of Bacteria

Indentification of a viral, bacterial, protozoan or fungal agent, pathogenic for *L. terrestris*, is the first step in developing a biomarker assay capable of correlating chemical induced suppression of the non-specific immune response with reduced host resistance to infection by a disease-producing organism. Of the disease-producing organisms listed above, bacteria were selected because of their availability, ease of maintenance and exposure protocols, literature available describing an association with earthworms, and lack of research concerning the association of viral, protozoan and fungal agents with *L. terrestris* or any other earthworm. Specific organisms, *A. hydrophila* and *P. aeruginosa*, were chosen for bacterial challenge experiments because they are known to be opportunistic pathogens for immunocompromised hosts, both vertebrates and invertebrates.

Neither *A. hydrophila* nor *P. aeruginosa* were found to be particularly pathogenic for *L. terrestris* at infective doses of up to 10^8 CFU/earthworm using either the oral or intracoelomic injection route of exposure. Hairi [3] reported 49% and 99% mortality in *E. foetida* after injection of a challenge dose of 10^7 and 10^8 *A. hydrophila* CFU/earthworm, respectively. This is significantly greater than the 10% mortality found in this study using *L. terrestris* injected with the same dose of the same organism. This may be explained by the fact that *L. terrestris* is physically a much larger species of earthworm, so a higher dose might be required to produce equivalent mortality.

Injection of *P. aeruginosa* at doses of 10^7 and 10^8 CFU/worm proved to be more pathogenic (30% mortality) than injection of *A. hydrophila* (10% mortality) at the same dose. Resistance to *A. hydrophila* is probably due to the earthworm's natural exposure to this organism which has been shown to be commonly found in soil [41] and as a result is a resident of the earthworm's coelomic cavity. Stein et al. [42] has demonstrated high concentrations of naturally occurring agglutinins against *A. hydrophila* in the coelomic fluid of both *L. terrestris* and *E. foetida*. Presence of these agglutinins can be explained by the natural exposure described above. Hairi [3] has demonstrated that coelomic fluid, collected from *L. terrestris* immunized with *A. hydrophila*, was able to inhibit 75.8% of the growth of this organism when compared to controls. *P. aeruginosa* has not been demonstrated to be an inhabitant of the earthworm's coelomic cavity. Thus, resistance to challenge with this organism should be expected to be somewhat lower, as demonstrated by results of this study.

Oral dosing did not prove to be an effective route of exposure as determined by mortality it produced. Bacteria were most likely cleared from the intestinal tract without colonizing.

Resistance to bacterial challenge found in this study may best be explained by the recent isolation and characterization of an antimicrobial peptide from the coelomic fluid of *Lumbricus rubellus*, an earthworm very similar in characteristics to *L. terrestris* [43]. Cho et al. [43] are currently studying the biology of this peptide they call Lumbricin I with the hope that it will form the bases for a new class of antibiotics. To date Lumbricin I has been characterized as a 29-amino acid peptide which exhibits bacteriocidal activity

against a broad spectrum of Gram-positive and Gram-negative organisms, in addition to viral and fungal agents. The existence of Lumbricin I, along with the better understood bacterial humoral factors present in earthworms, probably explains the high degree of resistance earthworms possess against bacterial challenge.

Bacterial Challenge Following Copper Exposure

Exposure to Cu^{++} at sublethal concentrations of 1, 2 and 3 μg Cu^{++}/cm^2 for 120-h prior to bacterial infection with either *A. hydrophila* or *P. aeruginosa* did not have a pronounced influence on the host resistance to challenge as measured by earthworm mortality.

Observations in this study suggest that Cu^{++} exposure resulted in a reduced ability of exposed earthworms to extrude coelomocytes when compared to unexposed control animals. These observations are supported by results reported by Giggleman [32] that described earthworms exposed to Cu^{++}, at the same concentrations used in this study, had a reduced ability to extrude cells, along with a reduced viability of those cells extruded. Additionally, Burch et al. [44] reported that in vitro exposure of coelomocytes to Cu^{++} at concentrations as low as 1 μg Cu^{++}/L resulted in coelomocyte death and lysis.

The results of research in this study, when combined with the findings of Giggleman [32] and Burch et al. [44], suggest that in vivo Cu^{++} exposure may have mediated coelomocyte death followed by autolysis . Autolysis would result in the release of a variety of bacteriostatic and bacteriocidal agents including lytic factors, agglutinins, lysozyme, and Lumbricin I, discussed above, into the coelomic cavity and coelomic fluid.

These agents, once released, could exert their actions on injected and intubated bacteria, resulting in elevated resistance to bacteria.

Coelomic Fluid Agglutination Activity Following Copper Exposure

Exposure to Cu^{++} at sublethal concentrations of 3 μg Cu^{++}/cm^2 for 120-h prior to coelomic fluid collection resulted in the production of RRBC agglutination titers that were significantly greater than those found for coelomic fluid collected from earthworms exposed to distilled water.

These results support the previously stated suggestion that in vivo Cu^{++} exposure caused coelomocyte death, autolysis, and release of the cell lysate into the coelomic cavity. Tissue concentrations of Cu^{++} in animals exposed to 3 μg Cu^{++}/cm^2 were significantly higher than those found in unexposed controls. Increased concentrations of agglutinins, as measured by increased titers, serve as an excellent indicator for coelomocyte lysis since these cells are the source of agglutinins. Additionally, these agglutinins serve as a marker for other antibacterial factors such as lysozyme, lytic factors and Lumbricin I, also synthesized and released from coelomocytes. Release of these factors, as demonstrated by agglutination titers, could explain the resistance to bacterial challenge in earthworms exposed to Cu^{++}.

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